Statement of Work

Background

SS1P is a recombinant immunotoxin based on the Pseudomonas exotoxin A (PE). PE is composed of three structural and functional domains. Domain Ia is responsible for cell recognition, domain II for translocation of PE across the cell membrane, and domain III for ADP-ribosylation of elongation factor 2, leading to arrest of protein synthesis and cell death. When only domains II and II are present, the truncated protein is termed PE38. The toxin is converted to the SS1P cancer therapeutic by replacing domain I with Fv portion of the SS1 monoclonal antibody (MAB) that binds strongly to mesothelin antigen present on many cancers, such as ovarian cancer, mesotheliomas, and pancreatic cancer. The Fv fragment constitutes the variable portion of the heavy chain linked to the variable portion of the light chain by the introduction of a novel disulfide bond. Use of the smaller Fv portion of the antibody potentially increases tumor penetration while maintaining specificity.

The heavy chain Fv of the SS1 MAB is cloned as a fusion at the N-terminus of the PE domain II (V_H-PE38). The light chain of the SS1 MAB Fv is cloned separately (V_L), and a cysteine residue is inserted into the framework region of each chain so the two chains can assemble into a disulfide-linked recombinant immunotoxin. The two proteins are expressed in E.coli from T7 based vectors inducible with isopropyl-β-D-galactopyranoside (IPTG) as originally described by Studier and Moffatt (J. Mol.Bio. 189 113 1986).

The two components of SS1P are expressed separately, inclusion bodies prepared, dissolved in guanidinium chloride containing a reducing agent, pooled, renatured, refolded (where the heavy and light Fvs combine, and then purified. Typical yields are 10% of the total protein present in inclusion bodies. The SS1P immunotoxin is stored frozen at -70°C.

There are 2 tasks in this statement of work and offerors may submit proposals for one of both of the following tasks:

TASK 1 PRODUCTION

1. Produce clinical grade cGMP SS1P drug substance.

TASK 2 FOMULATION

2. Formulate and vial cGMP drug substance to produce cGMP drug product.

The task of producing clinical grade GMP SS1P immunotoxin is composed of the following steps:

Fermentation; Cell Growth and Harvesting

1. Qualified Master Cell Banks for both the V_H-PE38 and V_L are obtained from the Laboratory of Molecular Biology (LMB), National Cancer Institute.

2. Qualified Working Cell Banks (WCB) for each is prepared. The Certificate of Analysis is reviewed by Quality Control and released to Manufacturing.

3. For light and heavy chain fermentations, a vial of each WCB is thawed and inoculated to seed flasks.

4. A seed flask near OD 2.0-3.0 is selected and inoculated to a 10 Liter fermenter. The optical density of the Seed Flask is recorded.

5. Induction with IPTG for 90 minutes is initiated when the OD_{600} is approximately 8-15. The optical density, agitation, pH, dissolved oxygen content, air flow temperature are recorded.

6. The Cell paste is then harvested by centrifugation, divided into 140 gram aliquots and is frozen at –70° C or below.

Inclusion Body Preparation

1. Thaw cell paste aliquots and resuspend in TES buffer (50 mM TRIS-HCl, 20mM EDTA, 20mM NaCl, pH 7.4).

2. Digest with lysozyme in TES buffer.

3. Disrupt released DNA with a tissue homogenizer.

4. Centrifuge to recover inclusion bodies.

5. Incubate in 2.5% Triton X-100.

6. Centrifuge to recover inclusion bodies.

7. Resuspend pellet and centrifuge three times with 2.5% Triton X-100

8. Resuspend pellet and centrifuge four times with 50/20 TE buffer (50 mM TRIS-HCl, 20 mM EDTA) to remove the Triton X-100 detergent.

9. Resuspend pellet in solubilization buffer (100 mM TRIS, 2 mM EDTA, and 7 M guanidine-HCl, pH 8.0).

10. Clarify solubilized inclusion bodies by centrifugation.

11. Remove sample for determination of protein concentration by Pierce Coomassie Plus Assay, and protein size determination by SDS-PAGE.

12. Dilute solubilized inclusion bodies to 10mg/ml and frozen at –70° C or below.

Refolding, Concentration and Buffer Exchange

Downstream purification processes are performed in a class 10,000 environment within a class 100 biological safety cabinet (BSC).

1. Thaw heavy (SS1VH-PE38) and light (SS1VL) chain inclusion body solutions.

2. Combine solutions of SS1VH-PE38 and SS1VL in a 2:1 weight ratio.

3. Reduce by adding dithioerythritol (DTE) to a final concentration of 10 mg/ml, mix well but gently and incubate for 16-24 hours at room temperature.

4. Dilute denatured protein solution 100-fold in refolding buffer (100 mM TRIS-HCl, 0.5 M L-arginine, 2 mM EDTA, 0.9 mM oxidized glutathione, pH 9.5).

5. Incubate for 42 to 46-hour period at 8-10°C to refold SS1P protein.

6. Concentrate refolded solution 10-fold by tangential flow filtration using a 10,000 MW cut-off membrane and refolding buffer without glutathione.

7. Continue tangential flow filtration with 20mM Tris-HCL pH7.4 and .1M urea until the conductivity of the solution reaches <7 mS.

8. Filter concentrated and dialyzed SS1P protein solution through a 0.2 micron filter.

Initial Purification

After the refolding step the correctly folded disulfide linked immunotoxin must be separated from impurities such as improperly folded immunotoxin, other insoluble bacterial proteins, RNA and DNA. We use two ion exchange steps that separate molecules based on charge. Q-Sepharose is an inexpensive media used to clean up the dialyzed refolding mixture. The Q-Sepharose column binds negatively charged molecules and positively charged molecules pass through. Most of the contaminants are removed, and the immunotoxin is concentrated. Source-Q ion exchange chromatography is used next to further purify the protein. In this step we use a linear NaCl gradient (0-500 mM) and observe immunotoxins eluting at a NaCl concentration between 250-300 mM; aggregated immunotoxin will elute at a higher NaCl concentration. Source-Q chromatography will concentrate the properly refolded immunotoxin.

1. Load concentrated protein solution onto a Q-Sepharose column and elute into 10 ml fractions.

2. Sample fractions for SDS-PAGE gel analysis.

3. Identify fractions enriched with specific immunotoxin and pool.

4. Determine protein content of the pooled fractions by the Pierce Coomassie Plus™ Assay.

5. Dilute each pooled batch four-fold, and load onto a 100 ml Source-Q Ion-Exchange column.

6. Elute the immunotoxin is using a linear salt gradient and collect 8 ml fractions.

7. Sample fractions for SDS-PAGE gel analysis.

8. Store fractions at –70˚C.

9. Identify fractions enriched with specific monomeric form of the immunotoxin from all Source Q batches and pool.

10. Dilute 5 fold with buffer.

11. Concentrate on Source-Q column.

Final Purification

Final purification is performed on a gel filtration column (Sephacryl S-200). This procedure serves to remove any aggregates and to exchange the immunotoxin into its final buffer formulation, Phosphate Buffered Saline (PBS).

1. Load concentrated immunotoxin solution onto a Sephacryl S-200 column, and elute 5 ml fractions.

2. Sample fractions for SDS-PAGE gel analysis.

3. Pool the fractions containing the monomeric form of the immunotoxin.

4. Determine the protein concentration of the pooled material with the Pierce Coomassie Plus™ Protein assay.

5. Filter the pooled material through a 0.2 micron filter into a storage container, and store at –70° C. This material is referred to as the **Intermediate Product.**

6. Test the intermediate product by SDS-PAGE, protein concentration by the Pierce Coomassie Plus™ Protein assay, Purity by SEC-HPLC, endotoxin, potency, appearance and description, pH, and bioburden.

Bulk Preparation

1. Thaw the Intermediate Product (S-200 pooled eluates from several runs), and pool these into a 2 Liter roller bottle.

2. Dilute to 0.9 mg/ml in PBS based on the protein concentration as determined by the Pierce Coomassie Plus™ Protein or A280 assay.

3. Filter the pooled and diluted bulk through a 0.2 micron filter into a sterile bottle, dilute to 0.7 mg/ml in PBS, and store at –70° C.

4. Release Test **Bulk Preparation** using the following tests:
    a. Protein concentration by A280 assay
    b. Protein concentration by Coomassie Blue dye binding assay
    c. Purity by SDS-PAGE (Coomassie Blue and Silver stain)
    d. SEC-HPLC
    e. Free thiol
    f. N-terminal sequencing,
    g. Endotoxin
    h. Appearance and description
    i. pH
    j. Sterility
    k. Potency on A431K5 cells
    l. Osmolality
    m. E.coli residual DNA content
    n. E.coli residual Host Cell Protein

The task of formulating and vialing cGMP drug substance to produce cGMP drug product is performed as follows:

1. Dilute SS1P drug product to 250 ug/ml in PBS (using starting concentration as determined by A280).
2. Sterile filter the solution using a sterile 0.22 micron Durapore membrane Millipak 200 filter.
3. Fill each sterile 10 ml Wheaton (serum, clear type I glass, 20 mm, part no. 223739) with 1.00 gm +/- 0.02 gm filtered SS1P solution.
4. Place a sterile stopper in each vial (West 20 mm, Teflon, 4416/50, grey, part no. 10144899) using a sterile stopper.
5. Place a sterile West seal (20 mm, flip off, part no. 54203028), on each vial and crimp.
6. Perform visual inspection on each container for particulate matter and flaws in the container-closure system.
7. Label each vial and each box of vials according to Cancer Therapy Evaluation Program (CTEP) National Cancer Institute (phone: 301-496-5725) specifications as follows:

SS1(dsFv)PE38 chimeric protein NSC 726388
215 mcg/vial (215 mcg/mL - 1 mL volume) Sterile
Store at -70°C Single use vial; vial contains no preservatives
Lot XXXXXXX Mfg Date: XX/XX/XXXX
Excipients:
Caution: New Drug Limited by Federal (USA) Law to Investigational Use
Mfg by: "Whatever Laboratory, Inc"
Distr. By: National Cancer Institute, Bethesda, MD 20892

8. Store the vials at -70 to -80 degrees Celsius.
9. Release test Drug Product using the following tests:
    a. Protein concentration by A280 assay
    b. Protein concentration by Coomassie Blue dye binding assay

c. Purity by SDS-PAGE (Coomassie Blue and Silver stain)
d. SEC-HPLC
e. Free thiol
f. N-terminal sequencing,
g. Endotoxin
h. Appearance and description
i. pH
j. Sterility
k. Potency on A431K5 cells
l. Amorality
m. Container-Closure Integrity

11. Ship vialed Drug Product on dry ice to Repository. Insure product to protect against in-transit damage to or loss of Drug Product.